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PITX1, a specificity determinant in the HIF-1 α -mediated transcriptional response to hypoxia

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Keywords: ChIP, HIF, Histone methylation, HIF-1 β , hypoxia, JMJC, JMJD2B, PITX1, transcription

Hypoxia is an important developmental cue for multicellular organisms but it is also a contributing factor for several human pathologies, such as stroke, cardiovascular diseases and cancer. In cells, hypoxia activates a major transcriptional program coordinated by the Hypoxia Inducible Factor (HIF) family. HIF can activate more than one hundred targets but not all of them are activated at the same time, and there is considerable cell type variability. In this report we identified the paired-like homeodomain pituitary transcription factor (PITX1), as a transcription factor that helps promote specificity in HIF-1 α dependent target gene activation. Mechanistically, PITX1 associates with HIF-1 β and it is important for the induction of certain HIF-1 dependent genes but not all. In particular, PITX1 controls the HIF-1 α -dependent expression of the histone demethylases; JMJD2B, JMJD2A, JMJD2C and JMJD1B. Functionally, PITX1 is required for the survival and proliferation responses in hypoxia, as PITX1 depleted cells have higher levels of apoptotic markers and reduced proliferation. Overall, our study identified PITX1 as a key specificity factor in HIF-1 α dependent responses, suggesting PITX1 as a protein to target in hypoxic cancers.

Introduction

Hypoxia, or changes in the oxygen supply, initiates a potent transcriptional program to allow cells to survive while attempting to restore oxygen homeostasis. As such, a great number of transcription factors are activated under hypoxic conditions¹, however, the hypoxia inducible factor (HIF) family, plays a crucial and central role in this transcriptional response.

HIF is a heterodimer composed of an oxygen-regulated α subunit and constitutively expressed β subunit. HIF- α subunits are regulated by oxygen via the action of a class of dioxygenases called prolyl-hydroxylases (PHD). PHDs require molecular oxygen, iron and 2-oxoglutarate as cofactors for catalyzing the hydroxylation of key proline residues in the oxygen-dependent degradation domain of HIF- α .² In addition to the regulation by PHDs, HIF's transcriptional activity is regulated by another type of dioxygenase enzyme called Factor Inhibiting HIF (FIH). FIH catalyzes the hydroxylation of an asparagine residue within the transactivation domain of HIF- α .³ This modification prevents binding of coactivators such as p300/CBP and thus inhibits HIF- α full transcriptional activation.^{4,5}

HIF activation leads to the upregulation of a variety of genes involved in angiogenesis, proliferation and autophagy. However,

not all genes are activated in the same cell at the same time, suggesting the presence of specificity determinants such as co-activators and co-repressors, or specific binding partners that direct HIF to particular promoters.^{5,6}

Hypoxia is an important component of many human diseases such as stroke and cancer. In particular HIF expression has been used as a prognostic marker in cancers such as colorectal and breast.^{7,8} Given the importance of this pathway in the pathology and response to treatment of cancer, identification of specificity determinants of the HIF response could lead to novel therapeutic approaches.

One protein that is often repressed in cancers such as colorectal and lung is the paired-like homeodomain pituitary transcription factor PITX1.^{9,10} PITX1 is required for pituitary gland and hind limb development.¹¹ It is also known to interact functionally with other transcription factors such as SF-1 and basic helix-loop-helix transcription factors.¹² More recently, PITX1 was identified in a screen for suppressors of RAS activity and tumor promotion,¹³ and also shown to repress TERT expression in melanoma cell lines.¹⁴

Here we show that PITX1 is a novel specificity determinant for HIF-1 α activity. Depletion of PITX1 leads to differential expression of a subset of HIF-1 α target genes and results in increased apoptosis in response to hypoxia. While most HIF-1 α targets were unaffected by PITX1, a number of JMJC protein demethylases were specifically

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altered. PITX1 was found to be important for the induction of JMJD2B, a histone demethylase with particular importance for breast and colorectal cancer progression. Furthermore, PITX1 reduction leads to changes in the proliferative ability of cells. Mechanistically, PITX1 regulates HIF-1 α activity by binding to HIF-1 β and regulating HIF recruitment to specific target promoters.

Results

PITX1 depletion results in higher HIF activity under hypoxic stress

To investigate if PITX1 plays a role in the molecular response to hypoxia, cells were depleted of PITX1 by siRNA and exposed to hypoxia for 24 hours. HIF transcriptional activity was assessed using luciferase reporter assays (Fig. 1A). In HRE-luciferase reporter cells, PITX1 depletion resulted in increased HIF transcriptional activity in U2OS and HeLa cells (Fig. 1A, Sup. Fig. S1A). To rule out off target effects of siRNA, we analyzed an additional siRNA oligonucleotide sequence targeting PITX1 (Fig. 1B). Cells were exposed to 1% O₂ for 24 hours prior to analysis of the luciferase assay. Under these conditions, PITX1 depletion using a different siRNA, also resulted in increased HIF transcriptional activity, indicating that the increased HIF transcriptional activity we detected with PITX1 depletion are not due to off target effects. Furthermore, PITX1 mediated effects on HIF dependent reporter activity were completely ablated when PITX1 was co-depleted with HIF-1 α (Sup. Fig. S1C) demonstrating the specificity of the reporters and that PITX1 depletion is altering HIF-1 α activity specifically.

As PITX1 is a transcription factor, it was possible that PITX1 depletion was causing a general transcriptional activation. To test this hypothesis, activity of a different transcription factor, NF- κ B, was assessed following a known activating stimulus, TNF- α (Fig. S1D). Under these conditions, PITX1 depletion did not result in any significant increase in NF- κ B activity, suggesting a degree of specificity for the effects observed.

We next determined if increased levels of PITX1 would have the opposite effect. U2OS cells were chosen due to lower endogenous levels of PITX1. Increasing amounts of PITX1 were transfected into U2OS-HRE luciferase cells prior to exposure to 1% O₂ for 24 hours. While lower levels of overexpression resulted in reduced HIF activity, higher levels of PITX1 expression resulted in a slight increase of HIF activity (Fig. 1C, Sup Fig. S1E). These results indicate that PITX1 has a threshold level that can interfere with HIF activity, suggestive of a role as a transcriptional modulator. It also rules out off target effects of the use of siRNA.

PITX1 modulates HIF activity via a post-transcriptional mechanism

As HIF activity is closely related to its expression levels, we next determined if changes in PITX1 resulted in changes in HIF-1 α mRNA. PITX1 depletion did not result in any significant change to HIF-1 α mRNA in both U2OS and HeLa cells (Fig. 1D, Sup. Fig. 1F), indicating that PITX1 modulating of HIF activity is post-transcriptional.

PITX1 depletion results in differential regulation of HIF-1 α targets under hypoxic stress

Our analysis revealed an increase in HIF-1 α transcriptional activity, when PITX1 was depleted assessed by reporter gene assay, with no change observed at the mRNA levels of HIF-1 α . To investigate if PITX1 depletion alters HIFs protein levels, we exposed cells to different times of hypoxia and analyzed HIF proteins by western blot (Fig. 2A). In the absence of PITX1, we did not detect any significant changes to the levels of HIF-1 α , HIF-2 α or HIF-1 β proteins in both of the cell lines tested.

U2OS cells have much lower protein levels of PITX1 when compared to HeLa cells (Sup. Fig. 2A), we used both cells to investigate the levels of HIF targets in the absence of PITX1 in response to hypoxia (Sup. Fig. S2B, C). Interestingly, while hypoxia can reduce the levels of PITX1 mRNA in U2OS cells, it has minimal effects in HeLa cells (Sup. Fig. 2B). In response to hypoxia, we did not observe any significant changes in a variety of HIF-dependent targets including PHD3, CA9 and GLUT1 (Fig. S2C).

One interesting class of HIF targets is the jumonji C Domain (JmJC) enzymes.⁵ Most of these are histone demethylases and hence by changing histone methylation patterns, and chromatin structure, can induce changes in gene expression quite rapidly.¹⁵ Depletion of PITX1 resulted in reduced levels of JMJD2B, and increased levels of JMJD1B, JMJD2A and JMJD2C (Fig. 2B, Sup. Fig. S2C, D). Interestingly, we also could detect a visible increase in the levels of histone H3 Lysine 9 trimethylation (H3K9me₃), following PITX1 depletion (Fig. 2B), a mark that is targeted by a variety of these JmJC proteins, including JMJD2B.^{16,17} Similar results were also obtained with an independent siRNA oligonucleotide targeting PITX1 (Fig. 2C). Importantly, increased levels of PITX1 achieved following overexpression of an exogenous plasmid, resulted in higher levels of JMJD2B and reduced levels of JMJD2C (Fig. 2D), while no change was observed for JMJD2A. These results support the notion of a dynamic regulation of JMJD2B and JMJD2C by PITX1.

As JMJD2B and PITX1 are deregulated in breast cancer,^{18–20} we also analyzed the effect of PITX1 depletion in breast cancer cell lines. Using the same reporter system as in Fig. 1, we analyzed MDA-MB-231 HRE-luciferase reporter activity in the absence and presence of PITX1 (Fig. 2E). As observed with U2OS and HeLa cells, PITX1 depletion resulted in enhanced HIF-reporter activity in MDA-MB-231 breast cancer cells. To determine if, in the context of breast cancer, PITX1 also controls JMJD2B expression, we also analyzed the effects of PITX1 depletion on JMJD2B protein levels in MDA-MB-231 and MCF-7 cells. In both these cell lines, PITX1 is required for JMJD2B expression following hypoxia (Fig. 2F and Sup. Fig. S2E).

PITX1 and HIF-1 α cooperate to control JMJC expression in cells

JMJD2B has been associated with the HIF response in a number of tissues.^{20,21} Furthermore, JMJD2B levels are de-regulated in a number of human cancers, suggesting that this is an important protein.²² Given our findings that PITX1 impacts on HIF-

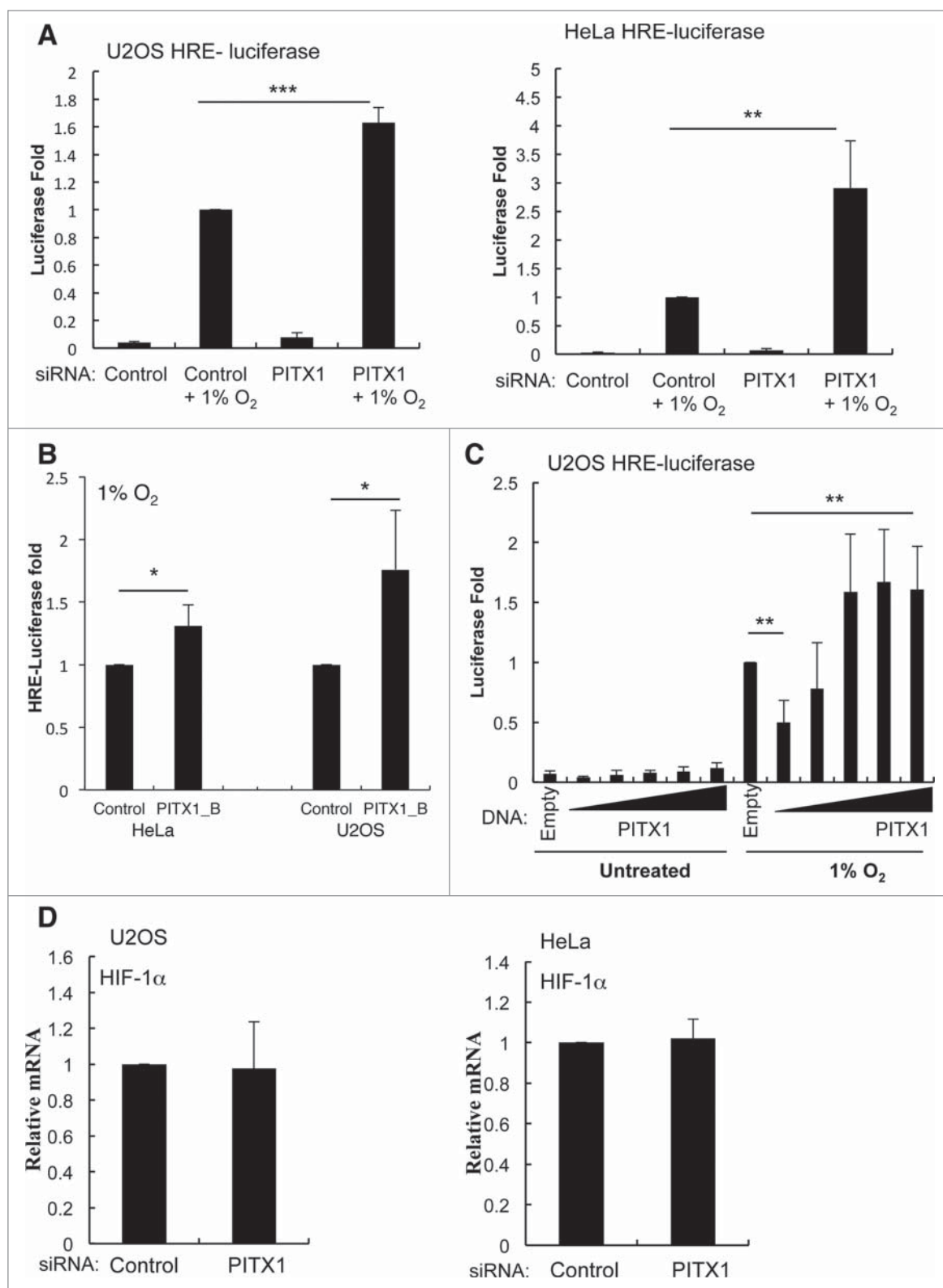


Figure 1. PITX1 levels control HIF transcriptional activity under hypoxic stress. **(A)** U2OS and HeLa cells stably expressing an HRE-luciferase reporter plasmid were transfected with control and PITX1 siRNA oligonucleotides prior to treatment with 1% O₂ for 24 hours. Luciferase activity was measured 48 hours post-transfection. Graph depicts mean and standard deviation of a minimum of 3 independent experiments. Student's t-test was performed to calculate p values, and levels of significance are denoted as follows: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. **(B)** U2OS and HeLa-HRE luciferase cells were transfected with control and PITX1_B siRNA oligonucleotides prior to treatment with 1% O₂ for 24 hours. Luciferase activity was measured 48 hours post-transfection. Graph depicts mean and standard deviation of a minimum of 3 independent experiments. Student's t-test was performed to calculate p values, and levels of significance are denoted as follows: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. **(C)** U2OS-HRE luciferase cells were transfected with

1 α transcriptional activity, we next determined the mechanism by PITX1 depletion altered expression of JmJC proteins. When PITX1 was depleted in U2OS and HeLa cells, we found that JMJD2B mRNA levels were significantly reduced both in normoxia and hypoxia (Fig. 3A, Sup. Fig. 3A, B). This analysis indicated that PITX1 is required for the expression of JMJD2B in both the cell lines analyzed. However, levels of JMJD2A and JMJD2C mRNA were increased when PITX1 was depleted in hypoxia, suggesting that PITX1 acts as a repressor of these genes (Fig. 3A, Sup. Fig. 3A, B).

Since some of the JmJC proteins are HIF-1 α targets, we next determined if the JmJC proteins we have analyzed were controlled by HIF-1 α in hypoxia (Fig. 3B). While JMJD2B and JMJD2C protein levels were reduced following HIF-1 α depletion, JMJD1B and JMJD2A proteins were increased in the absence of HIF-1 α (Fig. 3B). Given this dual function of HIF-1 α and PITX1, we investigated if PITX1 effects on JmJC protein expression was dependent or not on HIF-1 α . To this end, we performed co-depletion experiments where PITX1 and HIF-1 α were simultaneously reduced by siRNA (Fig. 3C). This analysis revealed that HIF-1 α depletion is dominant over PITX1 reduction. In other words, HIF-1 α co-depletion with PITX1 reversed the increased levels of JMJD2C, and did not further reduce JMJD2B protein expression. Similar results were also observed for JMJD1B and JMJD2A, 2 proteins that are increased in the absence of HIF-1 α or PITX1. In PITX1 and HIF-1 α co-depletion conditions, levels JMJD1B and JMJD2A proteins do not further increase, suggesting that PITX1 and HIF-1 α operate in the same regulatory pathway. Interestingly, this is also the case for the levels of H3K9me3.

H3K9me3 can be removed by several histone demethylases, such as JMJD2A, JMJD2B, JMJD2C.²³ Since we observed an increase in JMJD2A and JMJD2C but a decrease in JMJD2B, we wanted to understand how these enzymes interplay with each other to regulate this histone mark. To do this, we conducted siRNA mediated depletion of several these enzymes and analyzed the levels of H3K9me3. Our analysis revealed that JMJD1B is the most potent histone demethylase for H3K9me3 in HeLa cells, but that JMJD2B and JMJD2C also participate in this process (Fig. 3D and Sup Fig. S3C).

PITX1 modulates HIF-1 α activity by interaction with HIF-1 β

Thus far, we have established that PITX1 is a modulator of HIF-1 α transcriptional activity. However, our results suggest that this effect is not mediated via the control of HIF-1 α mRNA. We thus investigated if PITX1 was able to form a complex with HIF. To determine if this is the case, we performed co-immunoprecipitations for PITX1 and analyzed if HIF subunits were present in a complex in normoxia and hypoxia. Although,

we were unable to detect HIF-1 α interaction with PITX1, HIF-1 β was readily detectable in PITX1 immunocomplexes, under normoxic and hypoxic conditions (Fig. 4A). PITX1 interaction with HIF-1 β was also observed in co-transfection experiments, using tagged proteins, further supporting the notion that these 2 proteins interact (Fig. 4B). We could also confirm these biochemical results using immunofluorescence analysis (Fig. 4C), where quantification of colocalisation between HIF-1 β and PITX1 using Fiji software revealed a Spearman's rank correlation coefficient of 0.84 in normoxia or 0.82 in hypoxia. Finally, to demonstrate that indeed PITX1 interacts with HIF-1 β in cells, we employed a FRET approach using mCherry-PITX1 and GFP-HIF-1 β transfected cells (Fig. 4D, Sup. Fig. S4C). If 2 tagged-proteins interact, then, there is energy transfer from the donor fluorophore (GFP) to the acceptor fluorophore (mCherry). Based on this principal, if the acceptor is photobleached, one would expect an increase in the donor fluorescence intensity.²⁴ When, mCherry and GFP vectors were co-transfected into cells, acceptor photobleaching did not result in any measurable increase in GFP intensity as expected (Sup. Fig. S4C), indicating that mCherry and GFP do not normally interact in cells. However, when analyzing mCherry-PITX1 and GFP-HIF-1 β co-transfected cells, after mCherry-PITX1 photobleaching, a visible increase in GFP-HIF-1 β intensity was readily observed (Fig. 4D). FRET efficiency based on the level of increased GFP intensity, was measured at 49.9% (with standard deviation of 21.2), indicating that indeed PITX1 interaction with HIF-1 β is as robust as seen biochemically (Fig. 4A, B). Taken together these analyses demonstrate the strong interaction observed between PITX1 and HIF-1 β .

One possible explanation for the modulation of HIF activity, would be the possible disruption of HIF-1 α /HIF-1 β heterodimer in the absence/presence of PITX1. To test this hypothesis, we analyzed the levels of HIF-1 α /HIF-1 β complex formation in the presence or absence of PITX1. Under conditions of PITX1 depletion, HIF-1 α was able to readily complex with HIF-1 β (Fig. 4E). Again, we could confirm this observation using immunofluorescence for HIF-1 α and HIF-1 β , in the presence or absence of PITX1 (Fig. 4F). When we used Fiji to quantify the colocalisation between HIF-1 α and HIF-1 β , in presence or absence of PITX1, we could only detect a small reduction in the colocalisation statistics. Spearman's rank correlation coefficient for HIF-1 α and HIF-1 β colocalisation in control cells was 0.82, while in the absence of PITX1 this was 0.75. This suggests that PITX1 does not change HIF heterodimer complex in a significant manner.

Given, that we did not observe changes in all HIF-dependent genes, we hypothesized that PITX1 action was being sensed at specific HIF-dependent target gene promoters. To test this, we focused on the JMJD2B promoter. We performed Chromatin

Figure 1 (See previous page). empty vector or increasing concentration of PITX1 expression construct (0.1, 0.25, 0.5 1 and 2 μ g) prior to exposure to 1% O₂ for 24 hours. Luciferase activity was measured 48 hours post-transfection. For all graphs, data was normalized to control hypoxia treated sample. **(D)** U2OS and HeLa cells were transfected with control or PITX1 siRNA oligonucleotides for 48 hours prior to total RNA extraction. Levels of HIF-1 α mRNA were analyzed by qPCR. Graph depicts mean and standard deviation of a minimum of 3 independent experiments. Data was normalized using actin and compared to control siRNA.

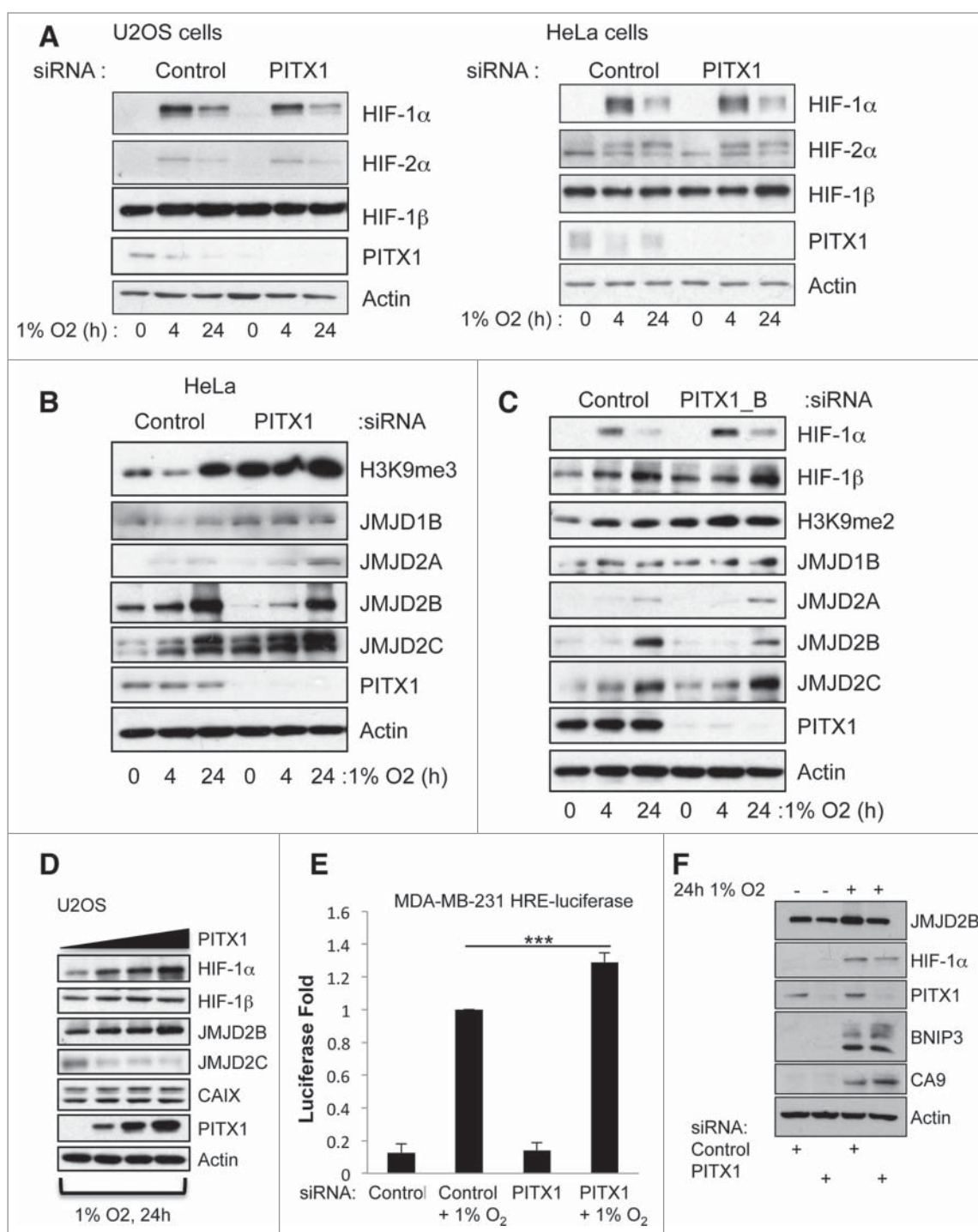
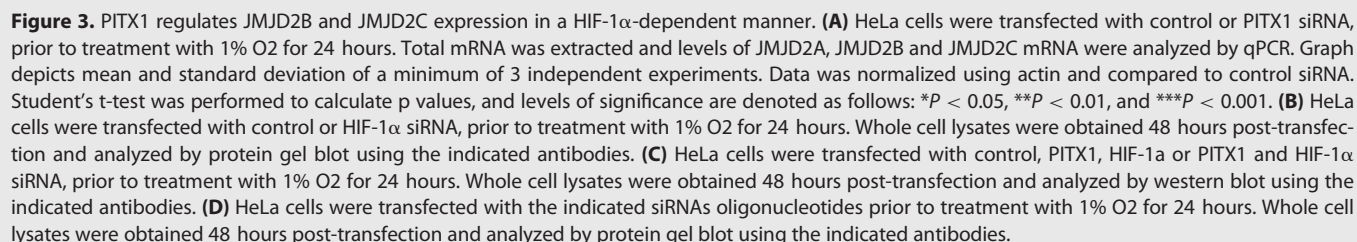


Figure 2. PITX1 is a specificity determinant for HIF-1a-dependent target gene activation. **(A)** U2OS and HeLa cells were transfected with control or PITX1 siRNA, prior to treatment with 1% O₂ for 24 hours. Whole cell lysates were obtained 48 hours post-transfection and analyzed by protein gel blot using the indicated antibodies. **(B)** HeLa cells were transfected with control or PITX1 siRNA, prior to treatment with 1% O₂ for the indicated periods of time. Whole cell lysates were obtained 48 hours post-transfection and analyzed by western blot using the indicated antibodies. **(C)** HeLa cells were transfected, processed and analyzed as in B. **(D)** U2OS were co-transfected with 1 μ g of GFP-HIF-1 β and increasing amounts of PITX1 plasmid (0.1, 0.25 and 0.5 μ g) prior to exposure to 1% O₂ for 24 hours. Whole cell lysates were obtained 48 hours post-transfection and analyzed by protein gel blot using the indicated antibodies. **(E)** MDA-MB-231-HRE cells were transfected with control and PITX1 siRNA oligonucleotides prior to treatment with 1% O₂ for 24 hours. Luciferase activity was measured 48 hours post-transfection. Graph depicts mean and standard deviation of a minimum of 3 independent experiments. Student's t-test was performed to calculate p values, and levels of significance are denoted as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. **(F)** MDA-MB-231 cells were transfected with control and PITX1 siRNA oligonucleotides prior to treatment to 1% O₂ for 24 hours. Whole cell lysates were obtained 48 hours post-transfection and analyzed by western blot using the indicated antibodies.



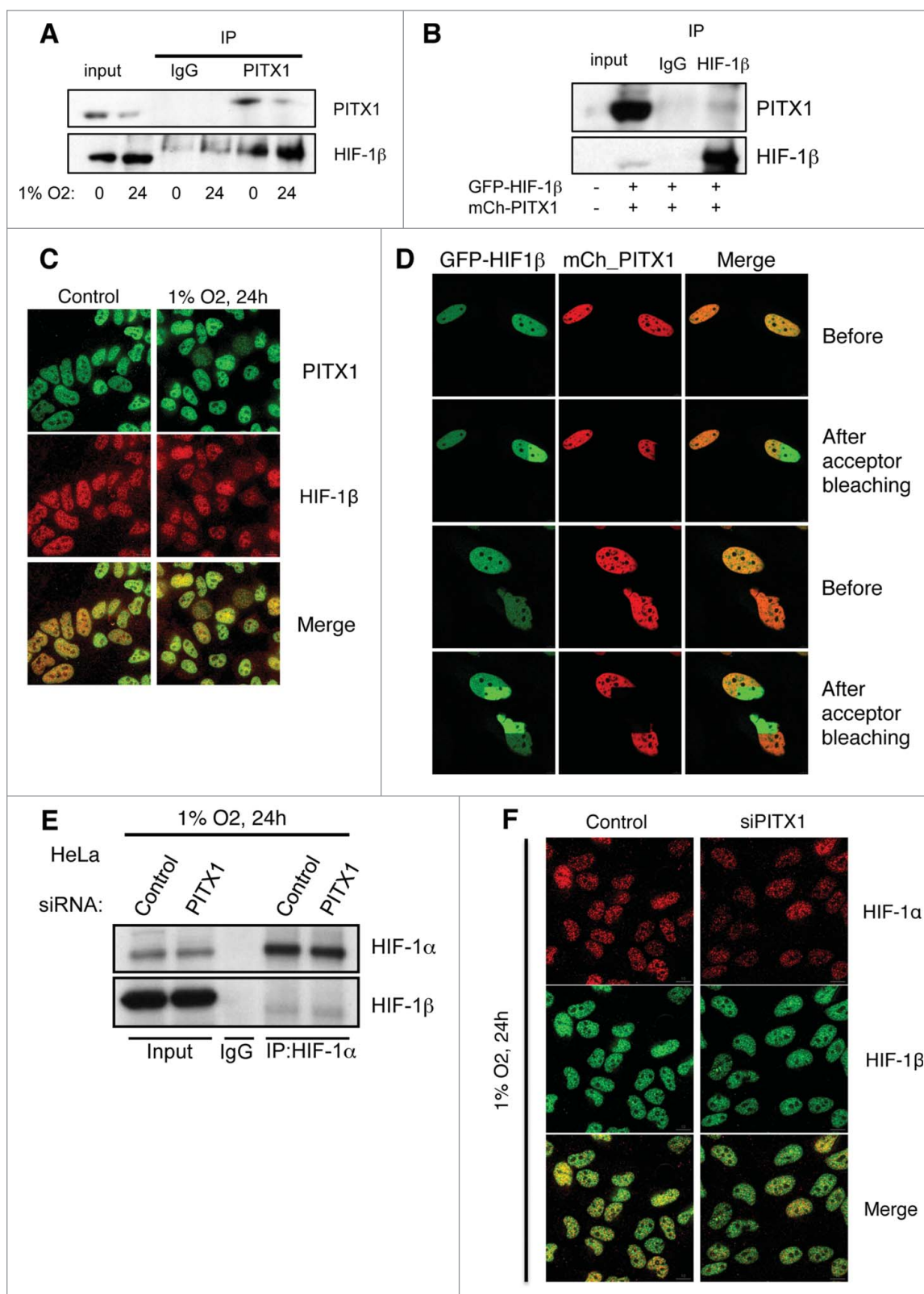


Figure 4. PITX1 binds HIF-1β but not HIF-1α and it does not disrupt the HIF-1α/HIF-1β heterodimer in a general manner. **(A)** HeLa cells were treated or not with 1% O₂ for 24 hours prior to whole cell lysis. 300 μg of protein were used to immunoprecipitate PITX1, using specific antibodies. Rabbit IgG was used as a negative control. Immunocomplexes were analyzed by western blot using the indicated antibodies. **(B)** Hek293 cells were transfected with 4 μg of empty vectors or GFP-HIF-1β and mCherry-PITX1 (mCh-PITX1) expression constructs for 48 hours prior to lysis. 300 μg of whole cell lysates were used to immunoprecipitate HIF-1β and normal rabbit IgG was used as a control. Immunocomplexes were analyzed by protein gel blot using the

Immunoprecipitation (ChIP) analysis for HIF-1 α and HIF-1 β , in the presence and absence of PITX1. ChIP revealed that in the absence of PITX1, there are significantly less HIF-1 β and HIF-1 α present at the JMJD2B promoter (Fig. 5A). This suggests that PITX1 is necessary to maintain the HIF heterodimer at the JMJD2B promoter. Interestingly, we could observe increased levels of HIF-1 β present at the JMJD2C and JMJD2A promoters but no significant change in the CA9 promoter (Fig. 5B). Furthermore, levels of HIF-1 α were not significantly altered in the JMJC2C and JMJC2A promoters (Fig. 5B). Finally, we investigated if increased PITX1 levels could alter HIF-1 β presence at the JMJD2 promoters analyzed. Given the difficulties of achieving similar levels of expression, we could only obtain statistically significant results for the JMJD2A promoter, where PITX1 overexpression results in reduced levels of HIF-1 β in normoxia (Fig. 5C). However, we could detect a tendency for increased levels of HIF-1 β at the JMJD2B promoter and a reduction in the JMJD2C promoter (Fig. 5C). These results are the opposite of those observed when PITX1 is depleted (Fig. 5A, B). Taken together, these results confirm that PITX1 confers gene selectivity in the HIF-1 transcriptional response via modulation of HIF-1 β presence at particular promoters.

PITX1 depletion results in changes of the cellular responses to hypoxia

Hypoxia can induce a variety of cellular responses depending on cell context.²⁵ To investigate the role of PITX1 in the cellular response to hypoxia, we started by analyzing markers of cell death/survival such as apoptosis and autophagy. In both U2OS and HeLa cells, when PITX1 was depleted and cells exposed to hypoxia, we observed increased levels of cleaved PARP, indicative of increased apoptosis. On the other hand, levels of p62, a protein essential for autophagy, and usually used as a marker of this process, were high in HeLa cells when PITX1 was depleted, but these still were reduced with hypoxia exposure (Fig. 6A). In agreement with this similar reduction, LC3 cleavage, another marker of autophagy was unchanged by the presence or absence of PITX1 (Fig. 6A). These results were also observed when cells were analyzed for the level of Annexin V staining using flow cytometry (Fig. 6B) and analysis of sub-G1 levels in cells cell cycle profile also detected by flow cytometry (Fig. 6C). Both these types of measurements indicate that PITX1 depletion results in increased levels of apoptotic cell death in HeLa cells.

Hypoxia has also been reported to induce changes in the proliferation and migration capacity of cancer cells.⁷ In HeLa cells, we could detect a small but significant increase in cell

proliferation following exposure to 24 hours of hypoxia (Sup. Fig. 5A). To address if PITX1 is involved in any of these responses, we analyzed the effects of depletion and overexpression of PITX1 in HeLa cells exposed to hypoxia in terms of their proliferation (Fig. 6D). While depletion of PITX1 results in a significant reduction in proliferation, overexpression of PITX1 resulted in higher proliferation levels in hypoxia (Fig. 6D).

To further determine the effect of PITX1 on cell proliferation, we analyzed the cell cycle profile of HeLa cells in the presence or absence of PITX1, treated or not with hypoxia for 24 hours (Fig. 6E; Sup. Fig. S5B). Apart from significant changes observed in sub-G1 cell numbers (Fig. 6C), we could also detect a significant reduction in the number of cells present in S-phase both in normoxia and hypoxia, when PITX1 is depleted (Fig. 6E). However, no additional significant changes could be observed in G1 or G2/M phases of the cell cycle, regardless of treatment (Sup. Fig. S5B).

The effect on cell proliferation/viability by PITX1 was also confirmed when colony formation potential was assessed (Fig. 6F). While, depletion of PITX1 resulted in a reduction in the number of colonies formed in hypoxia, overexpression produced the opposite effect (Fig. 6F, Sup. Fig. S5C). These results suggest that PITX1 is required for the proliferative capacity of cells, even in hypoxia.

Discussion

In this report we have investigated the role of PITX1 in the cellular response to hypoxia. We have found that PITX1 is a target-specific HIF-1 α modulator, resulting in differential expression of certain HIF-1 α targets following hypoxia. In particular, we have found that PITX1 is required for JMJD2B expression, an important histone demethylase often found deregulated in cancer and HIF-dependent target. Mechanistically, we found that PITX1 associates with HIF1 β and this is required for heterodimer loading at specific HIF promoters. Finally, we demonstrated that PITX1 is required for cell survival and proliferation of cells in hypoxia, suggesting that PITX1 could be a promising target for cancer therapy.

PITX1 was originally identified as a gene involved in pituitary gland gene expression,¹¹ as well as being important for mesoderm specification. In the mouse, deletion of PITX1 results in hindlimb impairment.²⁶ It has also been shown that PITX1 can have cell and gene-specific activities via its physical interaction with additional transcription factors.²⁷⁻²⁹ One of these studies identified NeuroD1/Pan1 as an interacting partner for PITX1.¹² NeuroD1 is part of the basic helix-loop-helix transcription

Figure 4 (See previous page). indicated antibodies. (C) HeLa cells were treated or not with 1% O₂ for 24 hours prior to being fixed and stained for immunofluorescence using the indicated antibodies. Images were acquired using a DeltaVision microscope, deconvolved and processed using OMERO client (opensource software). (D) HeLa cells were co-transfected with 1 μ g of mCherry-PITX1 (mCh-PITX1) and 1 μ g of GFP-HIF-1 β , as indicated, for 48 hours prior to fixation. Images were acquired before and after acceptor photo-bleaching as indicated in the methods. (E) HeLa cells were transfected with control or PITX1 siRNA, prior to treatment with 1% O₂ for 24 hours. Whole cell lysates were obtained and 300 μ g of protein were used to immunoprecipitate HIF-1 α , using specific antibodies. Mouse IgG was used as a negative control. Immunocomplexes were analyzed by western blot using the indicated antibodies. (F) HeLa cells were transfected with control or PITX1 siRNA, prior to treatment with 1% O₂ for 24 hours prior to being fixed and stained for immunofluorescence using the indicated antibodies. Images were acquired using a DeltaVision microscope, deconvolved and processed using OMERO client.

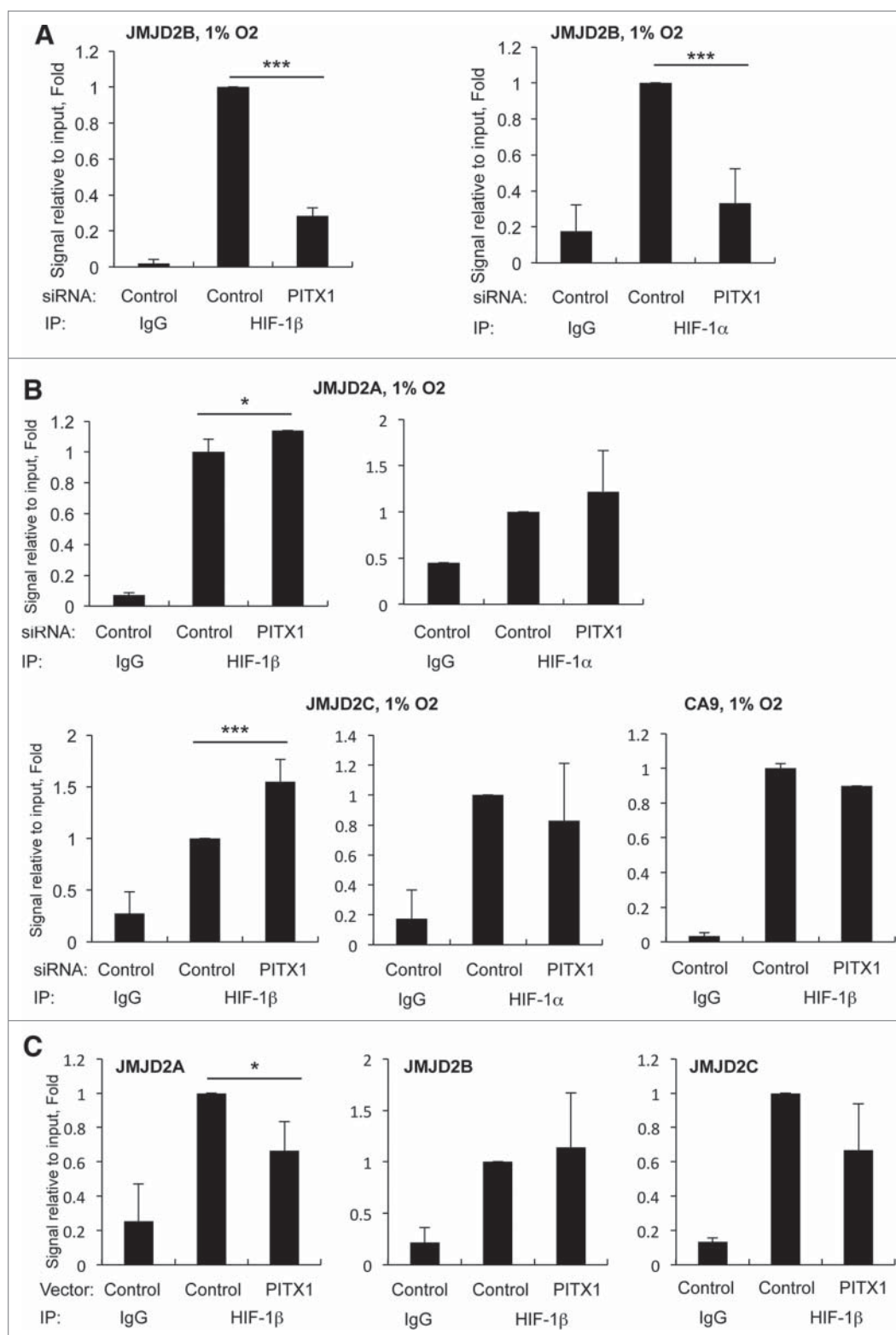


Figure 5. PITX1 controls HIF-1β levels at the JMJD2A, JMJD2B and JMJD2C promoters. **(A)** HeLa cells were transfected with control or PITX1 siRNA, prior to treatment with 1% O₂ for 24 hours. Cells crosslinked and processed for chromatin immunoprecipitation, using HIF-1α and HIF-1β antibodies. Levels of these transcription factors were analyzed at the JMJD2B promoter by qPCR. Graph depicts mean and standard deviation of a minimum of 3 independent experiments. Student's t-test was performed to calculate p values, and levels of significance are denoted as follows: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. **(B)** HeLa cells were treated and processed as in A, but levels of HIF-1β present at the JMJD2A, JMJD2C and CA9 promoters were analyzed. Levels of HIF-1α are also presented for JMJD2A and JMJD2C. Graph depicts mean and standard deviation of a minimum of 3 independent experiments,

factors group, which are transcription factors important for tissue identity.¹² Interestingly, HIFs are also bHLH transcription factors, but possess one additional domain, Per-Arnt-SIM (PAS).³⁰ Our results indicate that PITX1 binds to HIF-1 β , but we could not detect HIF-1 α consistently. While it is possible that this is due to antibody efficiency, our analysis is more consistent with the hypothesis that PITX1 preferentially binds HIF-1 β , since this interaction is observed in normoxia, a condition where HIF-1 α is not available. Additional studies, analyzing if this interaction is also observed in vitro, as well as domain mapping, would identify which domains are required for this complex formation.

PITX1 was found to be required to suppress RAS induced tumorigenesis; furthermore, several studies have shown that PITX1 expression is reduced in colorectal, prostate and lung cancer.¹³ However, PITX1 function has not been investigated in cervical or osteosarcoma types of cancer cells. Our analysis here, adds additional information regarding PITX1 function in these different cellular backgrounds. Interestingly, PITX1 is found overexpressed in breast cancer.^{10,31} PITX1 is responsive to estrogen, and is required for gene expression mediated by Estrogen receptor- α .³² HIF role in cancer has been extensively documented.^{7,33} However, different HIF- α isoforms have different functional outcomes for the cell.³⁴ HIF-1 α has been mostly studied and depending on the cancer/cell type it can be a tumor promoter or tumor suppressor.³⁴ Another interesting aspect is the temporal expression of the isoforms, with HIF-1 α being the first HIF to be induced and HIF-2 α being expressed after prolonged hypoxia exposure.³⁴

We have only analyzed the acute response to hypoxia, in the presence or absence of PITX1. As such, our results are related to HIF-1 α dependent gene expression. PITX1 reduction results in an increased HIF transcriptional activity, using a generic reporter assay. However, more detailed analysis revealed that only some targets are de-repressed in the absence of PITX1, while others actually require PITX1 for full hypoxia induction. It is also very likely that PITX1 can also regulate HIF-2 dependent genes due to its binding to HIF-1 β . Additional studies, investigating HIF-2 mediated responses will answer this question.

One interesting class of HIF-dependent genes that are altered by modulation of PITX1 is the JMJC demethylase enzymes. In particular, JMJD2B (JMJD2B) expression was particularly sensitive to PITX1 depletion. JMJD2B is a histone demethylase with preference for Lysine 9 when tri-methylated on Histone 3.³⁵ Furthermore, JMJD2B overexpression has been found in breast cancer and it is required for hormonal responsive cancer in this tissue.²⁰ Like PITX1,³² JMJD2B is also regulated by estrogen.¹⁹ Interestingly, we could also observe that PITX1 mediated regulation of JMJD2B in breast cancer cell lines such as MDA-MB-213 and MCF-7. More recently, JMJD2B has also been shown

to participate in colorectal cancer, via a HIF dependent mechanism.²¹ Given our result with PITX1, it is possible that targeting PITX1 or JMJD2B in hypoxia would be a valid novel therapeutic approach for hypoxic tumors.

We found that PITX1 has a differential effect on JMJC protein expression. While JMJD2B requires PITX1 and HIF-1 α , JMJD1B expression is regulated independently of HIF-1 α . On the other hand, JMJD2A and JMJD2C both require HIF-1 α for their expression and PITX1 effects on these proteins are also HIF dependent. We also determined, which of these enzymes has the most potent effect on H3K9me3 levels in HeLa cells. Our results identified JMJD1B as the most effective histone demethylase for this site in HeLa cells. Interestingly, JMJD2B and JMJD2C also have a contribution toward the regulation of this histone mark in these cells. It would be interesting to further investigate these effects on particular genes, as these enzymes might be specifically targeted to particular genomic loci. However, since these enzymes are relatively understudied, novel additional work will be necessary for the understanding of their biological functions in cells.

Materials and Methods

Cells

Human embryonic kidney Hek293, U2OS osteosarcoma, HeLa cervical cancer and MDA-MB-231 cell lines were obtained from the European Collection of Cell Cultures and grown in Dulbecco's Modified Eagle Medium (Lonza) supplemented with 10% fetal bovine serum (Gibco), 50 units/ml penicillin (Lonza), and 50 μ g/ml streptomycin (Lonza) for no more than 30 passages at 37°C and 5%CO₂. Stable cell lines U2OS-HRE-luciferase, HeLa-HRE-luciferase were described in³⁶ and maintained in 0.5 μ g/mL puromycin. MDA-MB-231-HRE cells were created as U2OS and HeLa-HRE cells, and also maintained in 0.5 μ g/mL puromycin.

Plasmids

CMV-PITX1 was obtained from Origene. GFP-ARNT (HIF-1 β) was a kind gift from Prof. Colin Duckett (Michigan, USA). mCherry-PITX1 was created by cloning PITX1 into mCherry-C1 (clontech). Vectors were sequenced to verify identity using our in house sequencing facility.

siRNA transfection

siRNA duplex oligonucleotides were synthesized by MWG-Eurofins and transfected using Interferin (Polyplus) per the manufacturer's instructions.

siRNA sequences are as follows:

Control, AAC AGU CGC GUU UGC GAC UGG³⁷;

Figure 5 (See previous page). except for JMJD2A, which presents the mean and standard deviation of 2 independent experiments. Student's t-test was performed to calculate p values, and levels of significance are denoted as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (C) HeLa cells were co-transfected with HIF-1 β and empty vector or PITX1 expression constructs for 48 hours prior to crosslinked and lysed. Chromatin immunoprecipitation was performed using the indicated antibodies. Levels of HIF-1 β were analyzed at the indicated promoters by qPCR. Graph depicts mean and standard deviation of a minimum of 3 independent experiments. Student's t-test was performed to calculate p values, and levels of significance are denoted as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

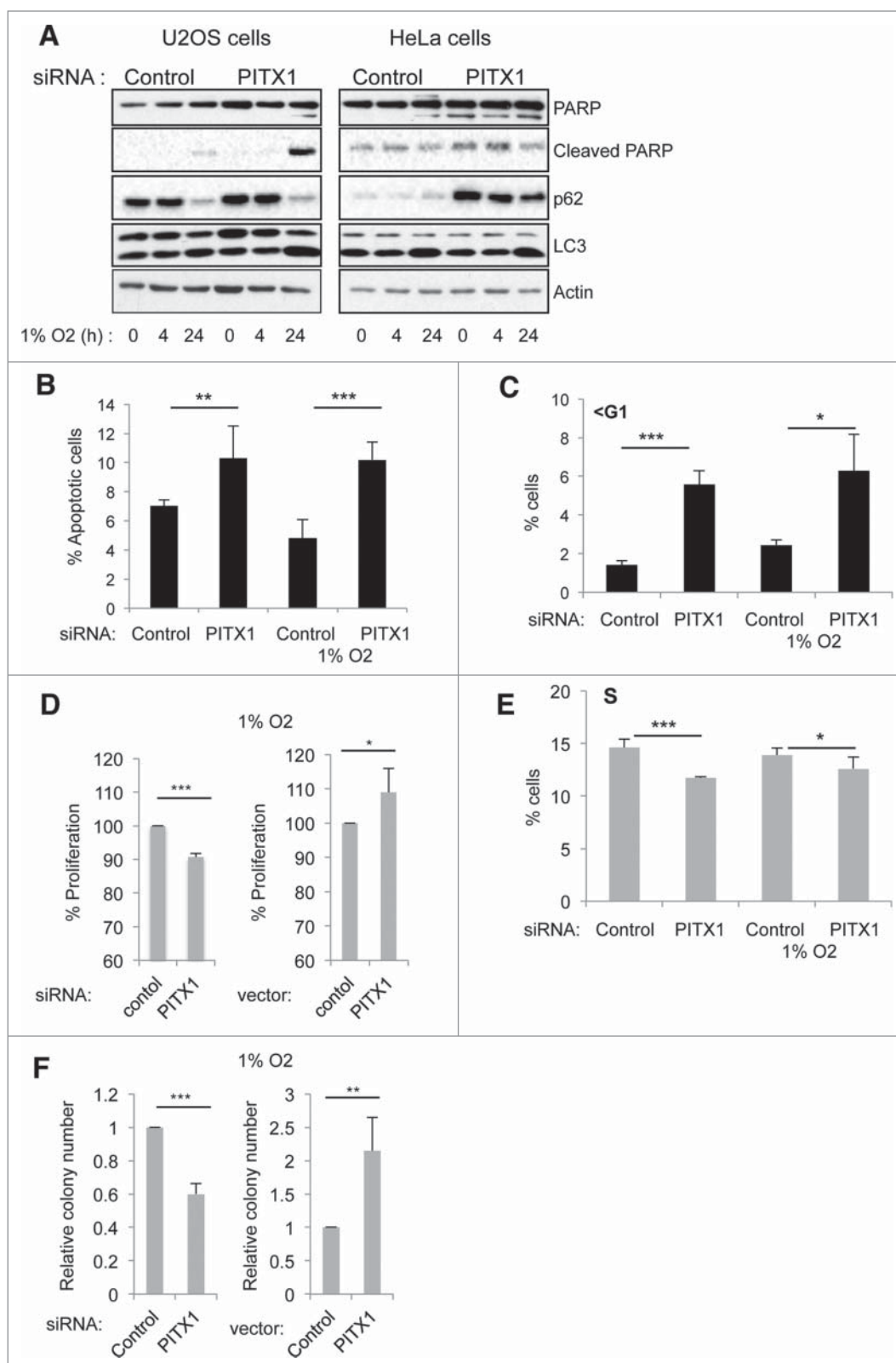


Figure 6. PITX1 regulates cell viability and proliferation in hypoxia. **(A)** U2OS and HeLa cells were transfected with control or PITX1 siRNA, prior to treatment with 1% O₂ for the indicated periods of time. Whole cell lysates were obtained 48 hours post-transfection and analyzed by protein gel blot using the indicated antibodies. **(B)** HeLa cells were transfected with control and PITX1 siRNA oligonucleotides prior to treatment with 1% O₂ for 24 hours. 48 hours after transfection, cells were trypsinized and analyzed for Apoptosis by flow cytometry. Graph depicts mean and standard deviation from a minimum of 3 independent experiments **(C)** HeLa cells were treated as in B, but cells were fixed prior to cell cycle analysis. Graph depicts mean a standard

HIF-1, CUG AUG ACC AGC AAC UUG A³⁷;
 PITX1, GCU UCA CCU UCU UCA ACU C
 PITX1_B, GAG CUU CAC CUU CUU CAA C
 JMJD2A, GAG UUA UCA ACU CAA GAU A
 JMJD2B, CGG CCA CAU UAC CCU CCA A
 JMJD2C, GGC GCC AAG UGA UGA AGA A
 JMJD1A, CUU AGU UGG UUC AGA AGU A

Hypoxia inductions

Cells were incubated at 1% O₂ in an in vivo 300 hypoxia workstation (Ruskin, UK), or in a H35 Don Whitley Scientific workstation. Cells were lysed for protein extracts, and RNA extraction in the workstation to avoid re-oxygenation. Whole cell lysates were obtained using a NP-40 lysis buffer (50 mM Tris pH8, 150 mM NaCl, 1% NP-40 (Calbiochem), 2 mM EDTA, 250 M Na₃VO₄, 1 mM DTT, 10 mM NaF and phosphatase inhibitors 1 tablet/10 mL (Roche complete)) RNA was extracted using PeqGOLD Total RNA Kit (Peqlab) as per manufacturer's instructions.

qPCR and PCR sequences

cDNA was made using QuantiTect Rev. Transcription Kit (Qiagen) and analyzed using Stratagene Mx3005p PCR machine and Brilliant II SYBR Green QPCR Low ROX Master Mix (600830, Agilent).

PCR primers are as follows:

HIF-1 α : For, CAT AAA GTC TGC AAC ATG GAA GGT and Rev, ATT TGA TGG GTG AGG AAT GGG TT;

β -actin: For, GTG GGA GTG GGT GGA GGC and Rev, TCA ACT GGT CTC AAG TCA GTG;

JMJD2A: For, TGT GCT GTG CTC CTG TAG and Rev, GTC TCC TTC CTC TCC ATC C;

JMJD2B: For, GGG GAG GAA GAT GTG AGT GA and Rev, GAC GGC TTT TGG AGG GTA AT;

JMJD2C: For, CGA GGT GGA AAG TCC TCT GAA and Rev, GGG CTC CTT TAG ACT CCA TGT AT.

Chromatin immunoprecipitation (ChIP)

Proteins were cross-linked with formaldehyde for 10 min. 0.125 mol/L glycine was added, and cells washed with phosphate-buffered saline. Cells were lysed with lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCL, pH 8.1, 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin), followed by sonication and centrifugation. The supernatant was precleared with sheared salmon sperm DNA and protein G-Sepharose beads (Sigma). The supernatant was incubated with specific antibodies overnight, and then with protein G-Sepharose beads for 1 h. After an

extensive wash step, the complexes were eluted with buffer (100 mmol/liter NaHCO₃, 1% SDS) and incubated with proteinase K. DNA was purified using QIAquick polymerase chain reaction purification kit (Qiagen). PCR was performed using the following primers:

JMJD2A: For, GGT CTC AAA CTG CCG ACT TC and Rev, CCC TGA GGG TCA TTA GGA CA;

JMJD2B: For, GGC AGT GAT TGG CAC CTG and Rev, GCC TCG CCT CTT CT TGG;

JMJD2C: For, GGG CAC CTT TAA GCT GTT TG and Rev, TGG GAA ATT TGG GAG ACT TG;

CA9: For, GAC AAA CCT GTG AGA CTT TGG CTC C; and Rev, AGT GAC AGC AGC AGT TGC ACA GTG.

Antibodies

Antibodies used were:

HIF-1 α (610958, BD Biosciences and sc-53546 Santa Cruz), HIF-2 α (PA1-16510, Thermo Scientific), HIF-1 β (3718, Cell Signaling; AT1199a, Abgent; sc-5580, Santa Cruz), β -actin (3700, Cell Signaling), p62/STQM (610832, BD Biosciences), PARP (9532, Cell Signaling), Cleaved PARP (9541, Cell Signaling), LC3B (3868, Cell Signaling), JMJD2A (3393, Cell Signaling), JMJD2B (8639, Cell Signaling), JMJD2C (PA5-23065, Thermo Scientific), H3K9me3 (9754, Cell Signaling), JMJD1B (5377, Cell Signaling), PITX1 (A300-577A, Bethyl Labs), CA9 (NB100-417; Novus Biologicals).

Immunofluorescence, image analysis and quantification

For immunofluorescence, cells were grown on coverslips and treated as indicated prior to fixation by incubation in 3.7% formaldehyde/PBS (pH 6.8) for 15 minutes. Cells were permeabilized in PBS-0.1% Triton X-100 for 15 minutes and then blocked in PBS-0.05% Tween-20 supplemented with 1% normal donkey serum for 30 minutes. Primary antibodies were used at the following dilutions: PITX1 (Bethyl labs) 1:50; HIF-1 β (Abgent) 1:100; HIF-1 α (Santa Cruz) 1:50, HIF-1 β (Santa Cruz) 1:100. Cells were analyzed and images were acquired using a DeltaVision microscope. Images were deconvolved and analyzed using OMERO client software (Open Microscopy Environment).³⁸ For colocalisation analysis, Fiji software was used and colocalisation levels were measured using Spearman's correlation coefficient value, where 1 signifies complete colocalisation and -1 signifies exclusion.

FRET analysis by acceptor photobleaching

Hela cells were co-transfected with mCh-PITX1 and GFP-HIF-1 β or respective empty vectors for 48 hours prior to fixation

Figure 6 (See previous page). deviation from a minimum of 3 independent experiments. **(D)** HeLa cells were transfected with control or PITX1 siRNA, or empty vector or 1 μ g PITX1 expression construct, prior to treatment with 1% O₂ for 24 hours. Levels of proliferation were measured using Alamar blue, 48 hours post-transfection. **(E)** HeLa cells were treated and in B, prior to cell cycle analysis by flow cytometry. Only S-phase data is presented in the graph that represents the mean and standard deviation from a minimum of 3 independent experiments. **(F)** HeLa cells were treated as in D, but processed for colony formation assay. Cells were stained and fixed 5 d post-transfection and colonies counted using ImageJ software. Graph depicts mean and standard deviation of a minimum of 3 independent experiments. Student's t-test was performed to calculate p values, and levels of significance are denoted as follows: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

with 3.7% formaldehyde. Cells were imaged in a Zeiss LSM 700 confocal microscope using a $\times 100$ Plan Apochromat objective (NA 1.46) and an optical section thickness of 0.7 μ m. The lasers (488 and 555) and detectors were set to image GFP and mCherry. Images of the same cells were acquired before and after mCherry-PITX1 photobleaching (100 scans of the ROI at maximum laser (555) power) using identical scan conditions. Acquired images were imported into OMERO. Donor intensities were measured using the ROI tool in OMERO. FRET efficiency was calculated by the following formula: $E = (I_{Dpost} - I_{Dpre}) / I_{Dpost}$, where ID is donor intensity pre and post bleaching.

Cell cycle and apoptosis analysis

Cells were transfected with siRNA oligonucleotides for 48 hours prior to fixation in 70% ethanol. Fixed cells were kept at -20°C for a minimum of 30 minutes. Cells were then washed in PBS, and stained with Guava Cell Cycle analysis solution from Millipore (4500–02200), and cells were analyzed in a Guava bench top flow cytometer.

Apoptosis analysis was performed on live cells, transfected with siRNA oligonucleotides for 48 hours prior to staining. Cells were resuspended in fresh completed growth media following trypsinization, and then stained with the Guava Nexin reagent (4500–0450; Millipore) for detection of apoptosis by flow cytometry. Cells were analyzed in a Guava bench top flow cytometer.

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Additional experimental procedures

Cell lysates were obtained as described in ³⁹ and using RIPA buffer. Western blot, colony formation assays, luciferase assays were previously described in ³⁹.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website. <http://www.tandfonline.com/kccy>

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